Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

PHYTOCHEMICALS AND ANTIOXIDANT ACTIVITY OF ULTRASOUND-ASSISTED AVOCADO SEED EXTRACT

(Fitokimia dan Aktiviti Antioksidan Ekstrak Biji Avokado Berbantukan Ultrabunyi)

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Received: 13 February 2022; Accepted: 14 March 2022; Published: 27 June 2022

Abstract

Avocado seed is the residue from direct food consumption and fruit processing industry, which is rich in phytochemicals. Ultrasound-assisted extraction (UAE) has been proposed as a cost-effective technique to recover the phytochemicals from plant fractions. Sonication time is one of the parameters that may influence the quantity and quality of phytochemicals isolated from the plant fractions when using UAE. Hence, this study aimed to investigate the effect of different sonication times (0, 20, 40 and 60 min) used in the UAE on the phytochemicals and antioxidant capacity of methanolic avocado seed extract. Results indicated the total phenolic, flavonoid and anthocyanin content of the seed extracts were in the range of 31.90-41.62 mg GAE/100 g, 8.25-12.51 mg RE/100 g, and 8.02-24.57 mg CGE/100 g, respectively. UAE for 60 mins generated the significant highest (p<0.05) amount of phenolic, flavonoid and anthocyanin content. Based on the antioxidant capacity tests, UAE for 60 mins resulted in the highest antioxidant activity.

Keywords: avocado seed, sonication time, phytochemicals, ultrasound-assisted extraction

Abstrak

Biji alpukat merupakan bahan sisa daripada penggunaan secara langsung dan/atau industri pemprosesan buah-buahan, yang kaya dengan fitokimia. Pengekstrakan berbantukan ultrabunyi (UAE) merupakan kos efektif teknologi untuk mengisolasi fitokimia daripada sumber tumbuh-tumbuhan. Dalam kaedah UAE, masa sonikasi adalah salah satu faktor yang mempengaruhi kuantiti dan kualiti fitokimia yang diisolasi daripada sumber tumbuh-tumbuhan. Kajian ini bertujuan untuk analisa kesan masa sonikasi yang berbeza (0, 20, 40 dan 60 minit) dalam UAE terhadap fitokimia dan kapasiti antioksidan ekstrak biji alpukat metanol. Hasil kajian menunjukkan jumlah kandungan fenolik, flavonoid dan antosianin dalam ekstrak biji alpukat adalah dalam lingkungan 31.90-41.62 mg GAE/100 g, 8.25-12.51 mg RE/100 g dan 8.02-24.57 mg CGE/100 g. Penggunaan masa sonikasi 60 minit dalam UAE dapat menghasilkan kandungan fenolik, flavonoid dan antosianin yang paling signifikan banyak (p <0.05). Berdasarkan

ujian kapasiti antioksidan, pengunaan masa sonikasi 60 minit dalam UAE dapat menghasilkan aktiviti antioksidan yang paling tinggi.

Kata kunci: biji alpukat, masa sonikasi, fitokimia, pengekstrakan berbantukan ultrabunyi

Introduction

Avocado, also known as *Persea americana* Mill. or alligator pear, is a tropical and subtropical fruit that has been gaining increasing worldwide demand in recent years due to its relevant nutritional values and health benefits. There are over 100 varieties of avocado fruits have been recorded in the database of California Avocado Society [1]. Hass variety avocado is one of the most widely sought for commercialization [2]. A major portion of Hass avocado is constituted by the pulp (65%) whereas the seed (20%) and peel (15%) make up the rest [3].

Avocado fruits have been primarily used for human consumption, and to a lesser extent, as raw material in the pharmaceutical and cosmetic industries [4]. However, only the pulp of avocado is valued for commercial usance while other fruit fractions such as the seed and peel are considered obsolete and usually throw away as waste products. Hass avocado seed is ovoid shaped with a length of 5 to 6.4 cm. The ivory-colored avocado seed is covered with a thin, brown-colored seed coat that adheres to the flesh cavity when ripe [5].

Proximate analysis done by Saavedra [6] revealed that the Hass avocado seed contained 52.7% of moisture, 2.51% of protein, 1.15% of ash, 1.11% of lipids, and 42.5% of nitrogen-free extract. The seed was also rich in phytochemicals such as phenolic acids, flavonoids and condensed tannins [7]. Based on current findings with regards to the biological effects of avocado seed extracts, they are known to have various ethnopharmacological properties for health-related conditions including anti-cancer, anti-inflammatory, anti-diabetic, anti-hypertensive, hypercholesterolemic, anti-microbial and the application in dermatological [8]. Hence, there has been a growing interest in the extraction of phytochemicals from avocado seeds to be utilized in the food and pharmaceutical industries.

Techniques such as maceration, infusion, heat extraction, enzyme-assisted extraction and Soxhlet extraction are commonly used to extract bioactive compounds from plants [9]. These techniques, however, have numerous limitations including the requirement of high extraction times, solvents and/or temperatures. Although advanced techniques like supercritical fluid extraction, microwave-assisted extraction and accelerated solvent extraction have demonstrated their efficiency in the recovery of phytochemicals, but these techniques are complex in operation and/or not cost-effective.

Ultrasound-assisted extraction (UAE) has been noted as one of the most effective techniques for the isolation of phytochemicals from plant materials [1, 9]. It utilizes acoustic waves to induce cavitation force to disrupt the plant cell wall, consequently enhancing the extraction rate of phytochemicals. Sonication time, solvent composition, sonication temperature, solid-solvent ratio and sonication frequency are the parameters that may influence the quantity and quality of phytochemicals isolated from the plant materials when using UAE [1, 9]. Among these parameters, sonication time was reported to have the greatest influence on the quality of plant extracts [10].

Industrial processing of avocado fruits produces a large number of seeds as wastes. Exploiting the phytochemicals of avocado seed waste may lead to new products and add value to the food industry. Therefore, this study aimed to investigate the effect of different sonication times on the UAE of phytochemicals and antioxidant activity of avocado seed extract.

Materials and Methods

Sample preparation

Hass variety avocado fruits were purchased from a local retailer in 2019. The fruits were kept at room temperature (25 ± 5°C) until ripe. Ripe avocado fruit,

as indicated by changes in peel color (from green to purplish-black) and gentle pressure felt when held in the palm, were manually separated into seed, peel, and pulp. The outer coat layer of the seed was peeled off before being cut into slices with an average thickness of 5 mm [6], followed by drying in a lab-scale freeze dryer (Liobras L101, Brazil). The lyophilized seeds were milled into fine powder by using an electrical blender (Panasonic MX-799S, Malaysia) and sieved through a 250 µm stainless steel sieve. The powdered sample was kept in an air-tight container at -5°C until use.

Chemicals and reagents

Analytical reagent grade of methanol, glacial acetic acid, hydrochloric acid (37%) was purchased from Fisher Scientific (Massachusetts, U.S.). Folin-Ciocalteu reagent, sodium carbonate, gallic acid, sodium nitrite, aluminium chloride hexahydrate, sodium hydroxide, rutin, 2,4,6-Tripyridyl-S-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron (III) chloride and iron (II) sulphate were purchased from Sigma–Aldrich (St. Louis, USA).

Ultrasound-assisted extraction

Approximately 1 g of avocado seed powder was mixed with 15 mL of 80% methanol in a 50 mL dark reagent bottle. The mixture was subjected to different sonication times (0, 20, 40 and 60 mins) in an ultrasonic bath [Elmasonic 100 H, Germany; 37 kHz ultrasonic frequency; 600 W ultrasonic output power; 11.8″ x 9.4″ x 5.9″ (L x W x H) internal dimensions] operated at a constant temperature of 30°C. Extraction time and ultrasonic temperature were regulated from the panel of the instrument. A centrifuge set at 4,000 rpm for 10 mins at room temperature was used to separate the methanolic seed extract from the powdered sediment. The extract was then transferred into a dark bottle using a pipette and kept at 4°C until use.

Phytochemicals

Determination of total phenolic content

The TPC was determined by the Folin-Ciocalteu method described by Tan and Azrina [11]. Briefly, 600 µL of methanolic seed extract was diluted with the same amount of distilled water in a test tube and subsequently added with 300 µL of 1 N Folin-Ciocalteu reagent and 600 µL of 2.0% Sodium carbonate. The mixture was incubated in the dark for 30 min. The absorbance was measured at 750 nm using a spectrophotometer (DLAB Scientific SP V1000, China) against a blank. A standard curve was plotted by using different gallic acid concentrations (0.20, 0.70 and 0.90 mg/mL) for TPC calculation. The results were expressed as mg gallic acid equivalent (GAE) per 100 g of samples on the basis of fresh weight (mg GAE/100 g).

Determination of total flavonoid content

The TFC was determined by the aluminium chloride colorimetric method described by Sharma and Agarwal [12]. Exactly 100 µL of methanolic seed extract was pipetted into a test tube containing 4 mL of distilled water and 0.15 mL of 5% sodium nitrite solution. After 6 min of incubation at room temperature, 0.15 mL of 10% aluminium chloride solution, 2 mL of 4% sodium hydroxide solution and 0.2 mL of distilled water were added into the mixture and vortexed. The mixture was allowed to stand for 15 mins. The absorbance was recorded at 510 nm using a spectrophotometer (DLAB Scientific SP V1000, China) against a blank. A standard curve was plotted by using different rutin concentrations (7.0, 9.0, 11.0, 13.0 and 15.0 mg/mL) for TFC calculation. The results were expressed as mg rutin equivalent (RE) per 100 g of samples on the basis of fresh weight (mg RE/100 g).

Determination of total anthocyanin content

The TAC was determined according to the method of Granato et al. [13]. Exactly 1000 µL of methanolic seed extract was pipetted into a 25 mL volumetric flask and diluted with distilled water to volume. The absorbance of the mixture was measured at 535 nm using a spectrophotometer (DLAB Scientific SP V1000, China) against a blank. The results were expressed as mg cyanidin-3-glucoside equivalent (CGE) per 100 g of samples on the basis of fresh weight (mg CGE/100 g).

Antioxidant activity

Determination of ferric ion reducing antioxidant power

The FRAP was determined according to the method of Bagheri et al. [14]. Freshly prepared FRAP reagent was obtained by mixing 2.5 mL of 10 mM TPTZ in 40 mM hydrochloric acid, 2.5 mL of 20 mM ferric chloride solution and 24 mL of 300 mM acetate buffer (pH 3.6). Then, 40 µL of methanolic seed extract was pipetted into 1.2 mL of the FRAP reagent and incubated at 37°C for 10 mins. The absorbance of the mixture was measured at 593 nm using a spectrophotometer (DLAB Scientific SP V1000, China) against a blank. A standard curve was plotted by using different concentrations of iron (II) sulfate solution (0.01, 0.03 and 0.06 mg/mL) for FRAP calculation. The results were expressed as mmol Fe^{2+} per kg of samples on the basis of fresh weight (mmol Fe^{2+}/kg).

Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity was determined according to the method of Ghafoor et al. [15]. Briefly, 0.25 mL of methanolic seed extract was mixed with 2 mL of 30 mg/L freshly prepared DPPH solution in methanol. The mixture was vigorously shaken and incubated at room temperature for 5 mins. A decline in absorbance was measured at 517 nm using a spectrophotometer (DLAB Scientific SP V1000, China) against a blank. Pure methanol was used as a negative control. The results were expressed as the percentage of antiradical activity (AA) of the extract on DPPH radicals relative to the control.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistic 26.0 for Windows (SPSS Inc., Chicago, USA). Data was summarized and expressed as mean ± standard deviation. Mean value refers to three analytical replicates. Comparison of mean differences among different sonication times (0, 20, 40 and 60 mins) was conducted using a one-way analysis of variance accompanied by Tukey's post hoc analysis. The association between bioactive compounds and antioxidant capacity was evaluated using Pearson correlation test. Statistical significance was set at $p < 0.05$.

Results and Discussion

Total phenolic, flavonoid and anthocyanin content

Various extraction media (methanol, ethanol, butanol, chloroform, ethyl acetate and water) have been utilized to extract the bioactive compounds from different fractions of fruits, either in pure form or in diluted form. In the present study, 80% methanol was selected as the media to prepare avocado seed extract. This is because 80% methanol has been deemed as a common medium to isolate the lipophilic and hydrophilic bioactive compounds of plant fractions [16].

The TPC, TFC and TAC of methanolic seed extracts sonicated at different times (0-60 mins) are shown in Table 1. Generally, increased sonication time enhanced the extraction of phenolics, flavonoids and anthocyanins from the avocado seed. This could be associated with the cavitation force produced by ultrasonic waves, which disrupt the integrity of plant cell walls and aid in the release of phytochemicals [9].

Phenolic compounds are secondary products produced by the metabolism of plants. They are widely recognized to have antioxidant characteristics, which could provide protection against oxidation induced by metal cations [7]. Avocado seed has been proposed as a potential food waste product for phenolic extraction [17]. It has been reported that the medicinal properties of avocado seed are attributed by its high phenolic compounds. As reported by Bahru et al. [18], the phenolic content of avocado seed (64%) was higher than its peel (23%) and pulp (13%). The TPC of methanolic seed extracts obtained from different sonication times was in the range of 31.90 to 41.62 mg GAE/100 g (Table 1). Application of UAE for 60 mins significantly increased ($p < 0.05$) the yield of phenolic content for 30%, as compared with the control without UAE at 0 min. Previously, the TPC of non-ultrasound assisted avocado seed extracts obtained from 70% methanol and 80% methanol were reported to be 2.92 mg GAE/100 g and 0.09-0.13 mg catechin equivalent (CE)/100 g, respectively [19, 20]. These values were much lower than the present study, possibly due to the different experimental protocols used in TPC analysis. As stated by Singh et al. [21], there are no standard method to determine phenolic compounds of plant seed

extracts and the parameters used (e.g. solute to solvent ratio, particle size of the seed samples and agitation rate) in the preparation of seed extract could affect the yield of TPC.

Flavonoid compounds are secondary metabolites corresponding to phenolics and are one of the most studied bioactive compounds due to their diverse benefits in human health. The TFC is analyzed based on color change due to the reaction between aluminium chloride and flavonoid compounds in the methanolic seed extract. Previous study reported the TFC of water and ethanolic avocado seed extract were 3.72 and 4.96 mg RE/100 mL, respectively [22]. These values were comparable with the results of the present study. In Table 1, application of UAE for 60 mins significantly increased (p <0.05) the yield of flavonoid content for 52%, as compared with the control without UAE at 0 min. A similar observation was reported by Ramić et al. [23], in which the TFC of ethanolic chokeberries extract was highest when sonicated for 60 min.

Anthocyanins are naturally occurring pigments that belong to the group of flavonoids. The flavylium cation form of anthocyanins requires a sufficiently acidic medium to maintain its stability, but not to the extent that can cause acyl moieties in acylated anthocyanins to be hydrolyzed [24]. It is difficult to achieve optimal protocol of extraction due to the diversity of anthocyanins structure and their sensitivity to heat, metal complexes, pH, and co-pigmentation. Hence, this study utilizes a method that quantifies TAC without requiring the use of chemical standards to plot a calibration curve. In Table 1, the application of UAE for 60 mins significantly increased (p <0.05) the yield of anthocyanin content for 102%, as compared with the control without UAE at 0 min. This may be due to the simultaneous enhancement of fragmentation and hydration from acoustic cavitation of UAE improves the mass transfer of anthocyanin compounds into the extraction solvent [9]. There has been limited data on the anthocyanin content of avocado seed extract documented thus far. Our current study demonstrated the effectiveness of UAE application in enhancing the yield of anthocyanin in avocado seed extract. Further study on quantifying the individual anthocyanin compound of avocado seed extract following UAE of 60 mins could be conducted.

Table 1. Phenolic, flavonoid and anthocyanin content of methanolic avocado seed extract

Sonication Time (mins)	TPC (mg GAE/100 g)	TFC (mg RE/100 g)	TAC (mg CGE/100 g)
0	31.90 ± 0.09[a]	8.25 ± 0.03[a]	8.02 ± 0.38[a]
20	32.20 ± 0.09[b]	8.74 ± 0.01[b]	16.04 ± 0.38[b]
40	32.26 ± 0.09[b]	8.81 ± 0.02[c]	21.26 ± 0.22[c]
60	41.62 ± 0.09[c]	12.51 ± 0.02[d]	24.57 ± 1.59[d]

Mean values in the same column with different letters are significantly different at p <0.05

FRAP and DPPH radical scavenging activity

Antioxidant capacity of methanolic seed extract from UAE was determined using two different antioxidant capacity assays and the finding results are shown in Table 2. Both FRAP and DPPH assays involved scavenging free radicals through electron transfer mechanism. As can be seen in both antioxidant capacity methods used, application of UAE at 60 mins had the highest antioxidant capacity, followed by 40 and 20 mins, while the control had the lowest antioxidant capacity. This shows the longest sonication time, the highest antioxidant activity in the methanolic seed extract. A similar observation was reported by Gómez et al. [25]. Their study demonstrated that the antioxidant activity of ethanolic avocado seed extract, as measured by oxygen radical antioxidant capacity assay, was the highest (158.77 mg Trolox Equivalents/g) when the longest sonication time (55 mins) was used during UAE.

Table 2. Antioxidant activities of methanolic avocado seed extract

Sonication Time (mins)	FRAP (mmol Fe^{2+}/kg)	DPPH (%)
0	0.72 ± 0.00^{a}	28.20 ± 1.42^{a}
20	0.96 ± 0.00^{b}	36.54 ± 0.83^{b}
40	1.19 ± 0.02^{b}	83.47 ± 0.19^{c}
60	1.72 ± 0.00^{c}	84.96 ± 1.20^{c}

Mean values in the same column with different letters are significantly different at $p < 0.05$

Correlation between phytochemicals and antioxidant activity

Correlation between phytochemicals (TPC, TFC and TAC) and antioxidant activities (FRAP and DPPH) are shown in Table 3. Positive correlation ($p < 0.05$) was found between total phenolic, flavonoid and anthocyanin content and antioxidant activity measured by FRAP and DPPH, indicating these phytochemicals contribute high antioxidant activity in the extract. This observation is in agreement with Wang et al. [26], who reported the high antioxidant capacity in Hass avocado seed extract was due to the high concentration of flavonoid and phenolic content. Sonication time is a significant factor that influences the extraction of plant phytochemicals and their antioxidant activity. This may be contributed by the cavitation effect of the ultrasound improved the fragmentation, swelling, hydration and pore production of the plant tissue matrix where the phytochemicals are to be isolated [27]. This will enhance the exposure of the phytochemicals and the extraction medium and help their release into the solvent, thereby increasing the antioxidant activity.

Table 3. Correlation between phytochemicals and antioxidant activity of methanolic avocado seed extract

	TPC	TFC	TAC	FRAP	DPPH
TPC	1	0.995**	0.675*	0.908**	0.609*
TFC		1	0.738**	0.940**	0.657*
TAC			1	0.916**	0.916**
FRAP				1	0.859**
DPPH					1

** Correlation was significant at $p < 0.01$

* Correlation was significant at $p < 0.05$

Conclusion

Avocado seed is the by-product of direct food consumption and food processing industry which could be further utilized to develop nutraceutical products. The TPC, TFC and TAC of methanolic seed extract were in the range of 31.90-41.62 mg GAE/100 g, 8.25-12.51 mg RE/100 g and 8.02-24.57 mg CGE/100 g, respectively. All phytochemicals and antioxidant activity demonstrated a positive and significant correlation. Efficacy of the UAE in terms of sonication time was demonstrated in this study. Sonication for 60 mins leads to the highest extraction yield of phenolic, flavonoids and anthocyanin content in methanolic seed extract. Further study to quantify the individual bioactive compound of avocado seed extract following UAE application of 60 mins could be carried out.

Acknowledgement

The authors would like to acknowledge Faculty of Science, Universiti Tunku Abdul Rahman, Kampar campus for providing the research facilities.

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